BINDING OF B[a]P DIOL-EPOXIDE (ANTI) TO NUCLEOSOMES CONTAINING HIGH MOBILITY GROUP PROTEINS

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1. Introduction

Recent experimental evidence has shown that when 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]P diol-epoxide (anti)), an important metabolite of B[a]P [1–5], was reacted with chromatin or nuclei, it bound preferentially to the internucleosomal DNA or linker region [6,7]. Only 15% of the total carcinogen bound to chromatin was found to be associated with the core histones [6,8], and in the case of chicken erythrocyte chromatin, histones H3 and H2B contained most of the bound carcinogen [9]. The very lysine-rich histones, however, did not show any appreciable binding of the carcinogen under non-dissociating conditions [9]. This specificity of binding to histones H3 and H2B appears to be a function of the three-dimensional structure of the nucleosome and the presence of the very lysine-rich histones.

Although the nucleosome, which is the fundamental repeating unit of the eukaryotic genome, has been well characterized (reviewed [10]), it has been suggested that several different nucleosomal particles exist [11–14]. These differences could be a function of microheterogeneity of some of the core histones because of amino acid substitutions [15] and/or post-synthetic modifications [16]. Alternatively, the structure of the nucleosome could be influenced by non-histone proteins, in particular the high mobility group (HMG) proteins [17], which have been found to bind to nucleosomes [18–22]. Current evidence suggests that the HMG proteins are associated with the active fraction of the eukaryotic genome in such a way that some of these proteins are responsible for confering DNase I sensitivity on the nucleosomes [21,22].

Since the three-dimensional structure of the nucleosome appears to dictate the binding of B[a]P diol-epoxide to the core histones, we have investigated the binding of this carcinogen to the fraction of chicken erythrocyte chromatin that contains the HMG proteins. Our results show that B[a]P diol-epoxide (anti) binds mainly to histones H3 and H2A of nucleosomes containing HMG proteins. Compared with [6,9], in which histones H3 and H2B were labeled in nucleosomes containing H1 and H5, these data suggest that the moderately lysine-rich histones H2A and H2B may be involved in the more dynamic aspect of the nucleosomal core particle.

2. Materials and methods

2.1. *Reaction of nuclei with B[a]P diol-epoxide*

After chicken erythrocyte nuclei were isolated, ^{14}C-Labeled B[a]P diol-epoxide (anti) (spec. act. 29.4 mCi/mmol) was added as in [6]. After incubation for 30 min at 37°C, the nuclei were washed with the isolation buffer (10 mM Tris (pH 7.0), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P40, 0.1 mM phenylmethanesulfonylfljoride (PMSF)) until the radioactive background was obtained.

2.2. *Isolation of HMG-containing nucleosomes*

Labeled nuclei as well as an unlabeled control sample were suspended in 100 ml isolation buffer;

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micrococcal nuclease (Worthington) was added (2 units enzyme/unit A_{260}), and the nuclei were digested for 90 min (approx. 35% acid solubility) at 37°C. At the end of the digestion the nuclei were precipitated by centrifugation (SS 34 Sorval rotor) at 1000 × g, 4°C for 10 min. The supernatant solutions were kept on ice, and the nuclei were washed 3 times with 10 mM Tris, 5 mM EDTA (pH 7.2). The supernatant solutions were combined, dialyzed against 0.2 mM EDTA, 0.1 mM PMSF, concentrated on an Amicon PM 10 membrane to 44.0 A_{260} units/ml, and then subjected to ultracentrifugation, through 5–25% linear sucrose gradients containing 0.2 mM EDTA (pH 7.2), 20 mM NaCl, 0.1 mM PMSF in an SW 25.2 rotor (Beckman) at 25 000 rev./min, 4°C for 24 h. The pooled nucleosomal fractions were dialyzed and recentrifuged through a similar gradient, except that an SW 40 rotor was used at 37 000 rev./min, 4°C for 12 h. The isolated nucleosomes were pooled and dialyzed against 50 mM Tris, 0.1 mM PMSF (pH 7.2).

2.3. *Polyacrylamide electrophoresis*

2.3.1. Protein gels

Aliquots of nucleosomes (0.5 A_{260} units) were removed, dialyzed against distilled water containing 0.1 mM PMSF, and lyophilized. The samples were dissolved in 25 μl sample application buffer [23] and electrophoresed in 15% SDS–polyacrylamide gels at 90 V for 6 h, or until the tracking dye (bromophenol blue) had reached the bottom of the slab gel. The gels were stained for 15 min in acetic acid–isopropanol containing 0.01% Coomassie blue R 250 [9]; they were destained in 10% acetic acid overnight. The next day the gels were rinsed for ~3 min in reagent grade dimethyl sulfoxide to remove all background stain, then resuspended in 10% acetic acid and photographed.

2.3.2. DNA gels

Double-stranded DNA was electrophoresed on 10% polyacrylamide containing 0.1% SDS in the gels as well as in the running buffer, whereas single-stranded DNA samples were electrophoresed on a 12.5% urea–polyacrylamide gel as in [24]. DNA gels were rinsed with 20% methanol for 5 min, stained with 0.1% toluidine blue for 30 min, destained overnight in water, and photographed with a Polaroid camera (Land 55 film).

2.4. *Fluorography*

The stained protein gels were treated for fluorography as in [25]. The X-ray films were exposed for 13 weeks at –70°C;

3. Results and discussion

Chicken erythrocyte nuclei were digested with micrococcal nuclease until ~35% of the nuclear DNA was rendered acid-soluble. However, the supernatant fraction, when measured directly in the presence of 0.01% SDS was found to contain 40–45% of the total nuclear DNA. Thus 5–10% of the DNA released into the supernatant after digestion by micrococcal nuclease was not acid soluble.

When this supernatant material was subjected to ultracentrifugation, the mononucleosomes (~11 S) that were isolated from peak II (fig.1) represented

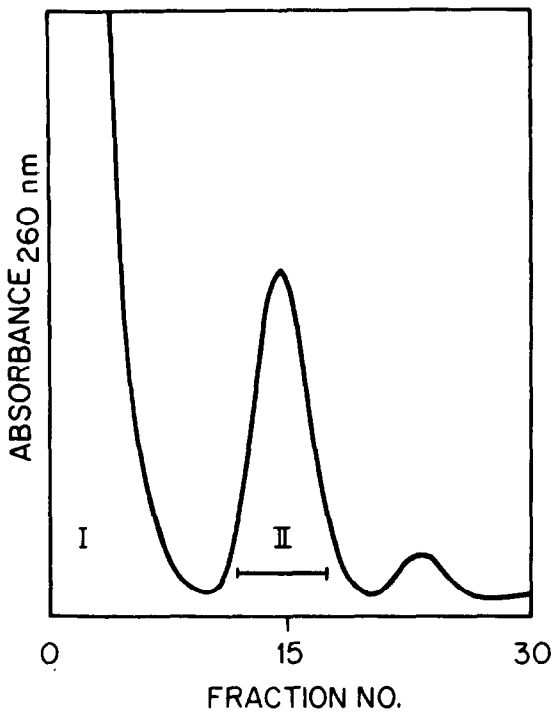

Fig.1. Absorbance profile of sucrose gradients of soluble fraction after extensive micrococcal nuclease digestion. Peak I contains subnucleosomal material. Peak II contains nucleosomes. The SW 25.2 gradients were fractionated with a Model 640 ISCO density gradient collector equipped with an ISCO UV-5 absorbance monitor. The profile shown is a representative profile of 6 gradients. Identical profiles were obtained from untreated and carcinogen-modified nuclei.

7% of the total nuclear DNA. To remove any possible subnucleosomal contaminants, the nucleosomal fraction was pooled, dialysed against 0.2 mM EDTA (pH 7.0), 0.1 mM PMSF, concentrated on a PM 10 membrane (Amicon) and re-isolated after ultracentrifugation (fig.2). This material which contained ~3% of the nuclear DNA was used for all subsequent analysis.

Examination of the protein gels showed that both the control and the B[a]P diol-epoxide labeled nucleosomes had a similar protein banding pattern (fig.3A). Further analysis on mini slab gels [26], with purified chicken erythrocyte HMG proteins as standards, showed that these nucleosomes contained a substantial amount of HMG, 2, E, and 14 proteins, while the proteins labeled 4 and 12 (fig.3B) could be minor breakdown products.

Analysis of double-stranded DNA gels revealed that these particles contain ~155 base pairs of DNA (fig.4A), whereas single-stranded DNA gels (fig.4B) showed the presence of two DNA bands of equal concentration, as judged from densitometer tracings (not shown). Therefore these particles either contain two distinct populations of discrete lengths of double-stranded DNA or they all contain a single-stranded section which is protected from digestion by the presence of the HMG proteins. These HMG con-

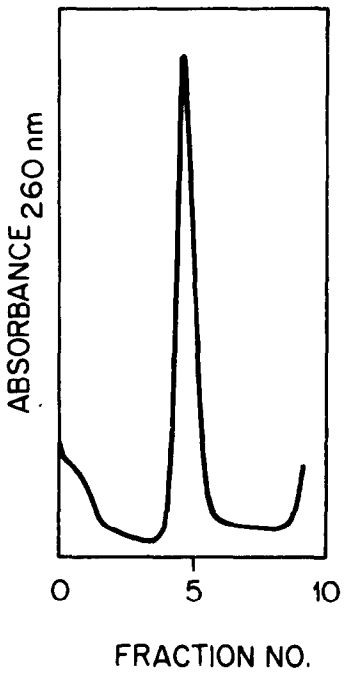

Fig.2. Sucrose density profile of nucleosomes isolated as in fig.1 (peak II) and recentrifuged on an SW 41 Ti rotor at 37 000 rev./min, 4°C for 12 h. Gradients were fractionated as described in fig.1.

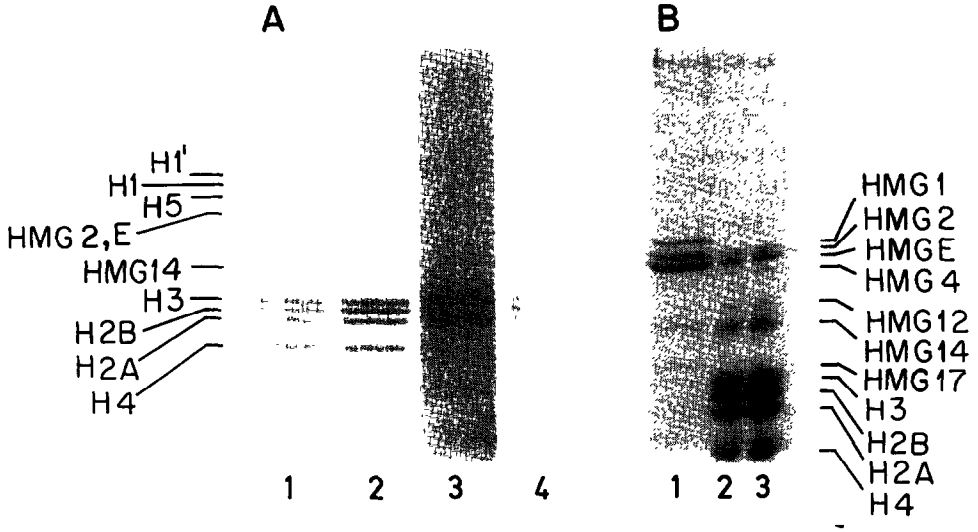

Fig.3. (A) 15% SDS–polyacrylamide protein slab gel. (1) Standard total histone proteins isolated from chicken erythrocyte nuclei. (2) Carcinogen-treated HMG-containing nucleosomal proteins. (3) Fluorogram of the stained proteins shown in lane 2 showing that histones H3 and H2A were associated with the carcinogen. (4) Control HMG-containing nucleosomes. (B) Mini SDS protein slab gels [26]. (1) Total HMG proteins from mature chicken erythrocytes. (2,3) Proteins from carcinogen-labeled and control nucleosomes, respectively.

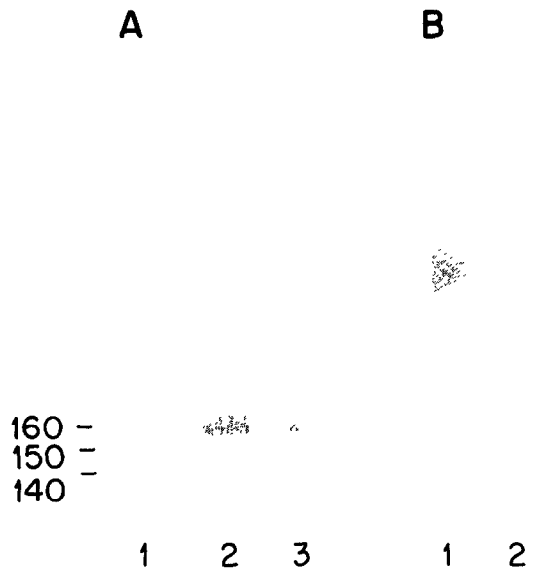

Fig.4. (A) Double-stranded gels. (1) KCl-insoluble nucleosomal DNA. (2,3) Carcinogen-labeled and control HMG-containing nucleosomal DNA, respectively. (B) Single-stranded DNA gels; (1) and (2) are single-stranded DNA fragments from carcinogen-labeled and control HMG-containing nucleosomes, respectively.

taining nucleosomes, isolated from mature chicken erythrocytes were largely devoid of histones H1 and H5. We have also found that the optimum conditions for the isolation of the HMG-containing nucleosomes is dependent on enzyme concentration and digestion time (in preparation). Although the problem of redistribution of the HMG proteins during digestion is a pertinent one, we like others [20] have no evidence to suggest that the HMG proteins have redistributed themselves. As pointed out in [22], it appears that in vitro HMG 14 and 17 have a very high affinity for that class of nucleosomes previously associated with the HMG proteins.

Since it has been shown that the HMG proteins are associated with the active fraction of the eukaryotic genome [12,20–22] the presence of HMG proteins in mature avian erythrocytes [30,31], together with the demonstration in this communication, that they are associated with nucleosomal particles, would suggest that the previously active fraction of the genome may still be associated with HMG proteins. Therefore, active transcription is probably not the only important factor that determines the presence of the HMG proteins.

In order to determine the distribution of the carcinogen, with respect to the histone proteins, the analysis of the fluorogram (fig.3A, lane 3) revealed that the carcinogen was predominantly associated with histones H3 and H2A in this fraction of nucleosomes containing HMG proteins. Since in [9] nucleosomes containing H1 and H5 the B[a]P diol-epoxide (anti) was mainly bound to H3 and H2B but not H2A under nondissociating conditions, it seems that the accessibility of histones H2A and H2B with respect to the carcinogen varies and that this change in differential binding is due to the presence of either the very lysine-rich histones (H5) or the HMG proteins.

Thus in nucleosomes associated with the very lysine-rich histones, in particular H5, histone H2A is protected from binding the carcinogen, whereas in nucleosomes containing the HMG proteins, histone H2B is protected. It seems, therefore, that unlike histones H3 and H4 the moderately lysine-rich histones H2A and H2B, which are the least conservative of the core histones [15,27,28] and are unable to reconstitute with DNA [10,29] in vitro into a nucleosome-like particle, may play a somewhat more dynamic role. Consequently, the association of histones H2A and H2B with the very lysine-rich histones or with the HMG proteins may have important consequences in the structural and functional aspects of chromatin.

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